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How does *Campylobacter* evade and/or avoid chicken's immune response?

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Declaration

I, Adelina (Adilya) Rakhimova have read and understood the University of Edinburgh guidelines on plagiarism and hereby declare that this written dissertation is all of my own work except where I indicate otherwise by proper use of quotes and references. This work has also not been submitted for any other degree or professional qualification.

Signed:

A. Rakhimova

Date: 30/04/2017

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Abstract

Campylobacter jejuni causes gastroenteritis in humans and is mainly acquired via consumption of contaminated poultry. *C. jejuni* is a frequent coloniser of chickens, often in the absence of overt pathology, yet produces a time- and magnitude- limited inflammatory response. One hypothesis is that glycosylation of surface moieties may mask the bacteria from recognition by the avian immune system, resulting in limited host responses. An *O*-linked flagellin glycosylation island (Δ*cj1321-cj1325/6*) has been previously shown to play a role in the colonisation of chickens.

Previously described methods for generation of avian antigen-presenting cells (APCs) were applied and tested. The objective of this project was to investigate the interactions between chicken APCs and *C. jejuni* strains that vary in legioniminic acid substitution of the flagellum (Δ*cj1321-cj1325/6* and Δ*cj1324*); Δ*cj1321-25/6* being a whole locus knock out, and Δ*cj1324* being a single gene knockout.

Primary bone-marrow derived APCs, macrophages (BMMs) and dendritic cells (BMDCs) were cultured for 6 days under standard conditions, then stimulated with either lipopolysaccharide (LPS, 100ng/ml), wild-type *C. jejuni* (11168H), mutant strains lacking genes responsible for flagellin *O*-glycosylation (Δ*cj1321-cj1325/6* and Δ*cj1324*) (10^8 CFU/ml) for 4 hours. Stimulated cells were then harvested and RNA was extracted. Changes in mRNA expression of pro-inflammatory cytokine chIL--1β were quantified Taqman RT-qPCR assay.

The mRNA expression levels of chIL-1β in BMMs, stimulated with mutant Δ*cj1321-cj1325/6* were significantly decreased p=0.042; no significant effect in chIL-1β mRNA expression (p=0.989) in BMMs stimulated with mutant Δ*cj1324* when compared to those stimulated with the wild-type.

The mRNA expression of chIL-1β in BMDCs, stimulated with the mutant Δ*cj1321-cj1325/6* was decreased (p=0.052). BMDCs stimulated with the mutant Δ*cj1324* have displayed a significant decrease (p=0.013) in chIL-1β, when compared to wild-type stimulated control (11168H).

Although the results presented are not conclusive of overall biological relevance, data appears to be indicative that *C. jejuni* flagellin O-glycosylation island may play a role in an immune response of chickens against *C. jejuni*, as there was notable decrease in expression levels of chIL-1β in both BMMs and BMDCs in response to the whole locus knock out mutant strain *cj1321-25/6.* This suggests that this locus might be important for host-bacteria interaction and, which in turn will allow *C. jejuni* colonisation to persist.

Contents

Chapter 1 - Introduction

Campylobacter-a problem on global scale

Genus *Campylobacter* consists of 16 species, majority of human infections (over 90%) are caused by *Campylobacter jejuni* and *Campylobacter coli* (Dasti *et al.* 2009). *C. jeuni* is a gram-negative spirally shaped bacterium, found ubiquitously in the environment. It is a major cause of human gastroenteritis in the developed world and has a significant financial impact, as it is estimated to be responsible for around 90% of hospital admissions due to food-borne illness in England and Wales in 2009 (FSA Chief Scientist report 2010-11). It is also a potentially life threatening infection (Young *et al.,* 2007; Nyati *et al.,* 2011). The symptoms of *C. jejuni* infection include bloody diarrhoea, vomiting, abdominal pain and fever, such symptoms usually self-limiting. Complications associated with *C. jejuni* may occur in some patients such as development of inflammatory bowel disease (Dasti *et al.* 2009) and autoimmune neuropathies such as Guillain-Barré and Miller Fisher syndromes which are caused by production of cross-reactive antibodies; it is estimated that approximately 1 in 1000 patients would develop Guillain-Barré syndrome (Ang et al. 2002, Semchenko *et al.,* 2010; Hardy *et al.,* 2011). *Campylobacter* infections have now replaced *Salmonella* infections as the leading cause of human gastroenteritis, and are still on the rise in Europe (scientific report of EFCA and ECDC, 2012). One of the main routes of acquiring the infection is via the consumption of contaminated poultry, with 60-80% of reported cases being due to poultry consumption. Up to 80% of retail poultry has been reported to be contaminated by *C. jejuni* (Zoonoses Report 2010). Control of *Campylobacter* colonisation in poultry is challenging, especially as legislation now prohibits the prophylactic use of antibiotics as growth promoters, which may have previously kept pathogen colonisation at lower

numbers (Dibner and Richards, 2005; Huyghebaert *et al.,* 2010). There is therefore a need for alternative methods for infection control in chickens and other farm animals (Kaiser, 2010; Huyghebaert *et al.,* 2010).

Campylobater in chickens, not a commensal.

Campylobacter colonisation of chickens was long thought to be a commensal relationship and, unlike human infection, does not result in apparent pathology and is frequently asymptomatic (Shane, 1992; Hendrixson and DiRita 2004; Young *et al.,* 2007; Bingham-Ramos *et al.,* 2008; Pajaniappan *et al.,* 2008; Conlan *et al.,* 2011). A number of exceptions to asymptomatic carriage exist, with a few reports of *C. jejuni*-associated neuropathies (Li *et al.,* 1996; Nyati *et al.,* 2011), association with avian vibronic hepatitis (Burch, 2005; Jennings *et al.,* 2010), and the ability to cause diarrhoea and systemic infection in young chicks (Sanyal *et al.,* 1984). A number of reports have shown that *C. jejuni* infections of chickens result in cytokine and chemokine production in chicken cell lines (Smith *et al.,* 2005; Borrmann *et al.,* 2007; Larson *et al.,* 2008; Li *et al.,* 2008) and in *in vivo* infection models (Smith *et al.,* 2008), triggering the adaptive immune response (Huang *et al.,* 2007; Shoaf-Sweeney *et al.,* 2008). Attachment to intestinal epithelial cells and *Campylobacter* invasion has been demonstrated (Byrne *et al.,* 2006; van Deun *et al.,* 2008; Hermans*et al.,* 2011), along with invasion of deeper tissues such as liver and spleen (Hofreuter *et al.,* 2008; van Deun *et al.,* 2008). Furthermore it has been identified that some *C. jejuni* strains result not only in mild, but relatively severe pathology, such as prolonged intestinal inflammation, diarrhoea and inflammation associated pathology in legs and feet of the birds (Humphrey *et al*. 2014). Knowledge about the interactions between *C. jejuni* and specific components of the avian immune system is, however, limited.

Innate immune system and clearance of pathogens.

The immune system is responsible for capture and clearance of pathogens and is superficially divided into two components, innate and adaptive. Innate immunity is responsible for the initial detection of pathogens and, in mammals, is performed by means of specific components: cells (neutrophils, macrophages, dendritic cells (DCs)), proteins (complement, antimicrobial peptides), pattern recognition receptors (PRRs) (Toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLR), nucleotide oligomerization domain (NOD)-like receptors (NLR), and C-type lectin receptors (CLRs)) (Kawai and Akira, 2011; Wells *et al.,* 2011).

In chickens, antigen capture and presentation is performed by DCs and macrophages, and partially by thrombocytes and heterophils both of which phagocytose pathogens (Wu and Kaiser, 2010). Pattern recognition receptors such as TLRs and NLRs are present, although there are different repertoires of each, whereas RLR is absent from the genome (Kaiser, 2010).

Antigen presenting cells (APCs) of the immune system are the link between innate and adaptive immunity and are responsible for antigen capture, processing, and presentation to appropriate T cell subsets in order to activate a suitable adaptive response. DCs are professional antigen presenting cells. In mammals the common myeloid progenitor in bone marrow differentiates under the influence of various cytokines, notably granulocyte-macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF or CSF-1) into naïve DCs and macrophages respectively (Gordon, 2003).

DCs in mammalian models were initially derived from bone-marrow progenitors ($CD34^+$) using GM-CSF and interleukin-4 (IL-4) (Lardon *et al.,* 1997). These cytokines were successfully cloned, characterised and expressed in the chicken (Avery *et al.,* 2004), and subsequently used to generate and characterise chicken primary bone-marrow derived DCs (Wu *et al.,* 2009). Following the capture and processing of antigen, mammalian DCs undergo a maturation process as they migrate to lymph nodes, making them less efficient at capturing antigens but enabling them to present antigens and stimulate T cells (Banchereau and Steinman, 1998). Chickens do not possess lymph nodes, however chicken DCs still undergo maturation (Wu *et al.,* 2009). Maturation of DCs results in morphological changes, and can be assessed by levels of expression of co-stimulatory molecules such as CD40 and CD86 (Banchereau and Steinman, 1998). In chickens, as in mammals, CCR6 is expressed on primarily immature DCs, whereas CCR7 is expressed on mature DCs (Wu *et al.,* 2011).

Macrophages, like DCs, can be derived from the bone marrow progenitor $CD34^+$ cells under the influence of M-CSF (CSF-1) or GM-CSF (Gordon, 2003). In chickens, the use of recombinant CSF-1 results in the growth of pure macrophage cultures from bone marrow precursors (Garceau *et al.,* 2010). Macrophages phagocytose pathogens and subsequently present their antigens to cells of the adaptive immune system. After encountering pathogens, macrophages become activated and can produce a variety of antimicrobial molecules such as nitric oxide (NO), reactive oxygen species (ROS), scavenger receptors (which aid in phagocytosis), pro-inflammatory cytokines and chemokines, and co-stimulatory molecules (Gordon, 2003).

Interaction between host's PRRs, such as Toll-like receptors (TLRs) and microbial pathogen-associated molecular patterns (PAMPs) plays an essential role in an innate immune

response. TLRs are able to detect highly conserved PAMPs, such as flagella (Smith *et al.,* 2003) and lipopolysaccharide (LPS) (Kawai and Akira 2011, Kumar *et al.,* 2011).

Recognition of PAMPs by TLRs leads to activation of the Toll/IL-1 Receptor (Resistance) domain (TIR) of PPRs, which activates downstream signalling (O'Neill, 2008) (Figure 1). Generally TLR downstream signalling involves recruitment of adaptor protein myeloid differentiation factor 88 (MyD88) and various members of the interleukin-1 receptor associated kinase (IRAK) family, which in turn phosphorylate members of tumour necrosis factor receptor (TNFR)-associated factor (TRAF) family and the resulting cascades then activate the release of nuclear factor κB (NF-κB) from the inhibitor NF-κB kinase complex (IKK), which results in activation of NF-κB, and subsequent transcription of cytokines, chemokines and co-stimulatory molecules (Kawai and Akira, 2004). Signalling pathways of human TLRs are summarised in figure 1.1. Signalling pathways in chickens are present and majority key features are conserved, like activation of NF-κB (Kannaki *et al.* 2010).

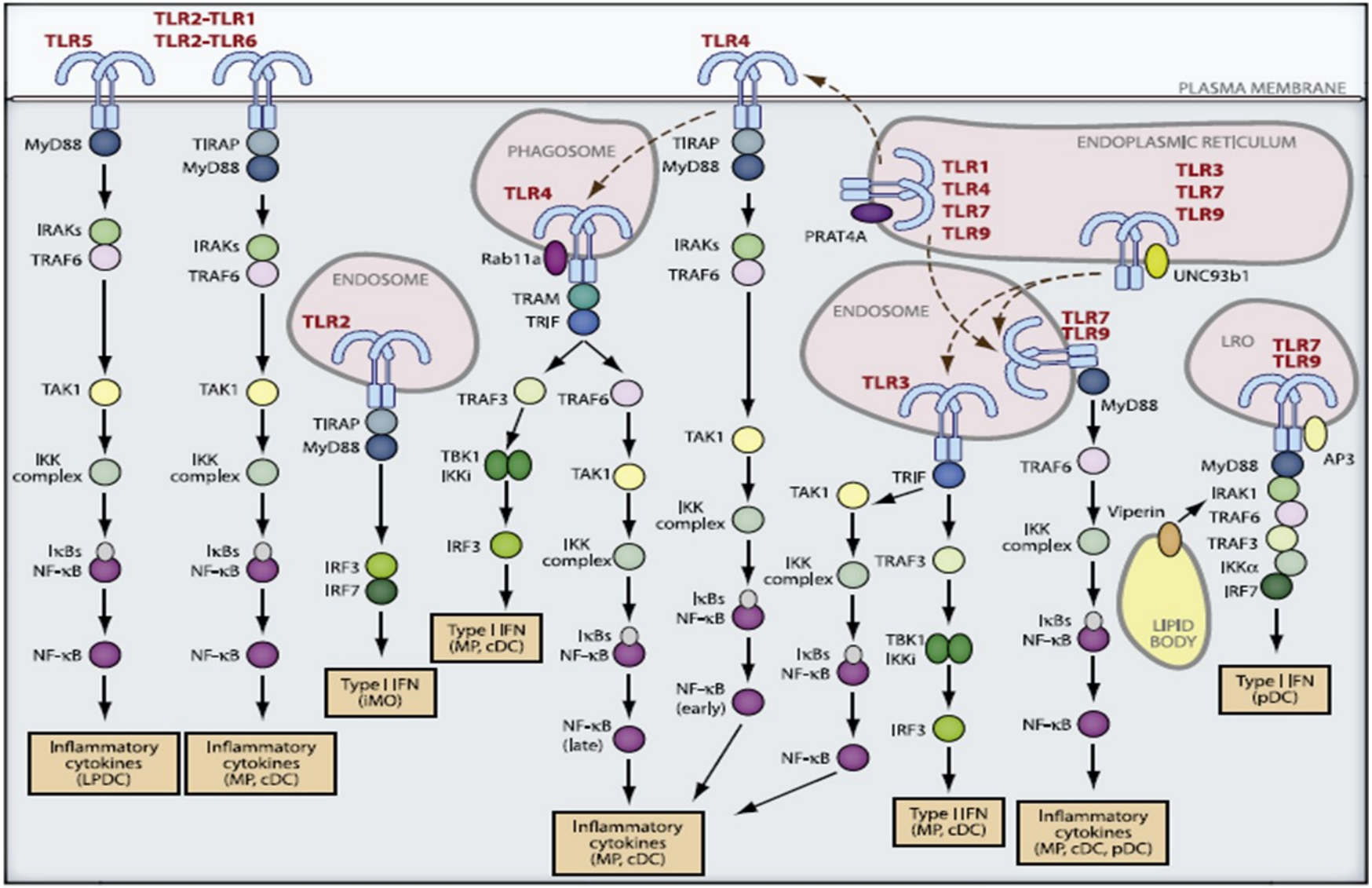


Figure 1.1 Summary of mammalian Toll-like receptors (TLRs) signalling in response to ligand interaction. Interaction with the ligands (PAMPs) results in the recruitment of adaptor molecules such as MyD88 and TIRAP. This in turn activates downsteam signalling involving IRAK, TRAF and IKK complex, which will eventually result in activation of NF-κB (Kawai and Akira 2011). Macrophages - MP, conventional DCs - cDC, plasmacytoid DCs - pDC, lamina propria DCs - LPDC, inflammatory monocytes – iMO.

Glycosylation of surface proteins by prokaryotes.

Glycosylation is one of the most common post-translational modifications, originally only attributed to eukaryotic organisms, now has been discovered and described for prokaryotes (Nothaft and Szymanski 2010). This process involves a covalent attachment of carbohydrate molecule to an amino acid (Valguarnera *et al.* 2016). Different types of bacterial glycosylation, which differ in the bond formed to form glycoproteins, these include *O*-linked: which involves attachment hydroxyl oxygen of serine or threonine (Ser/Thr) to carbohydrate; *N*-linked, which involves amide nitrogen of asparagine (Asn) linkage to carbohydrate; *S*-linked – attachment to the sulfur of cysteine (Cys), *C*-linked (*C*-mannosylation) an attachment α-mannopyranose to tryptophan (Trp) through a C–C linkage and surface layer (S-layer) protein glycosylation – covalent linking of glycans to surface of extracellular proteins (Schäffer and Messner 2017, Nothaft and Szymanski 2010,Tan *et al*. 2015).

Glycosylation has an important role in many of bacterial functions, such as adhesion, cell charge and protein stability (Howard *et al*. 2009, Tan *et al*. 2015). Bacterial glycosylation has also been associated with antigenic diversity in bacterial pathogens, avoiding of detection by TLRs. The glycosylation in eukaryotic organisms plays an important role for innate and adaptive immunity (van Kooyk and Rabinovich 2008), some bacterial species have developed an ability to mimic or use the host glycans like sialic acid (Rabinovich *et al.* 2012), and furthermore human commensal *Bacteroides spp* can use host derived glycans for more exclusive relationships (Tytgat and Lebeer 2014). Other roles for glycosylation include complex host-bacteria interactions (Tan *et al*. 2015).

***Campylobacter* immune evasion strategies.**

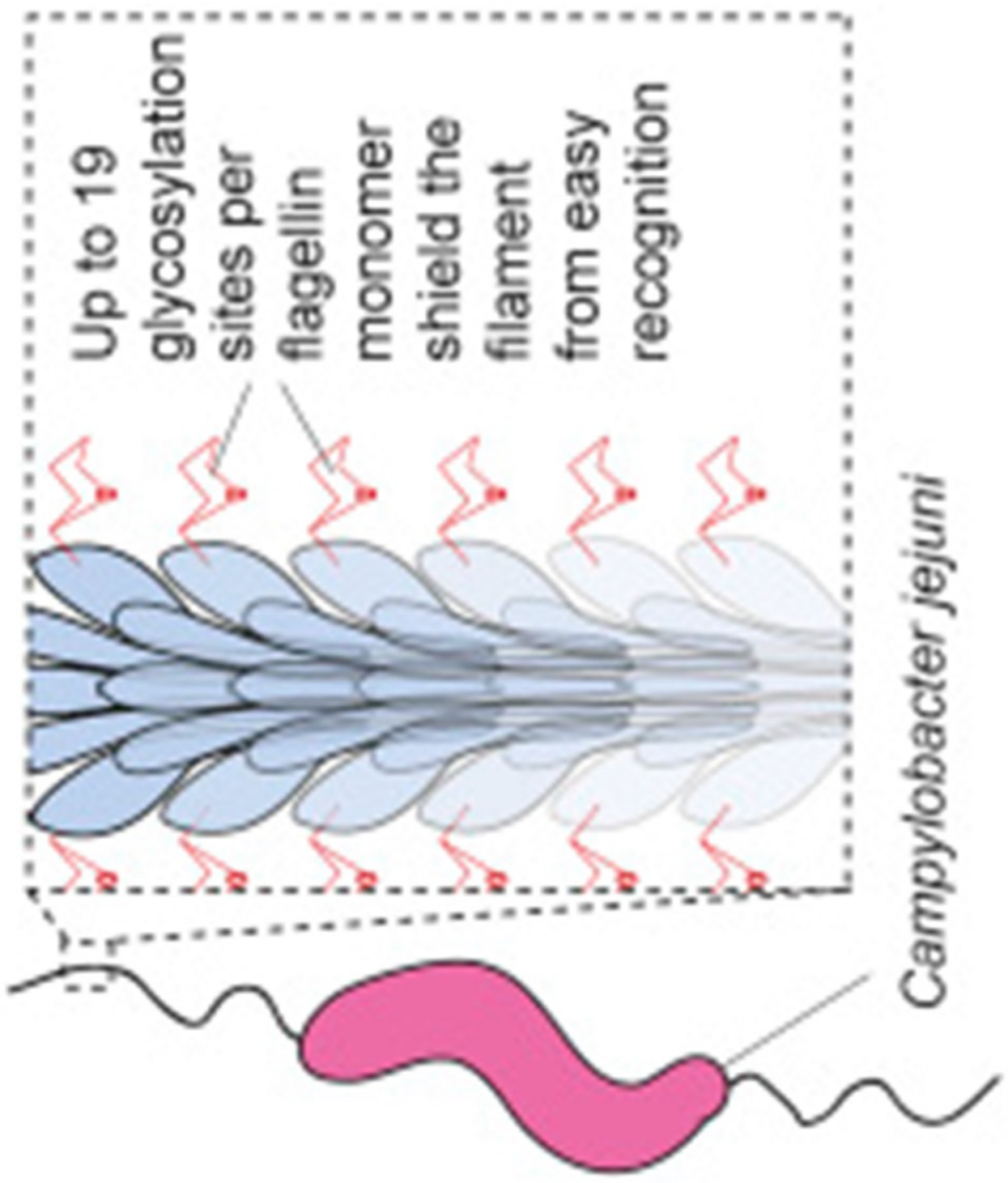
Campylobacter spp have an array of well-described immune evasion strategies. Variation of surface s-layer sugars has been helping immune evasion (Thompson 2002). A typical bacterial LPS consists of lipid A, core oligosaccharide, and O chain polysaccharide. *C. jejuni* surface LPS consists of lipid A and inner regions of core oligosaccharide (Penner and Aspinall, 1997; Morgan, 2010), and is referred to as lipooligosaccharide (LOS), some bacterial species LOS often replaces LPS, to avoid host immune response, generated against O-antigen or detection by TLR4 (Trent *et al.,* 2006; van Mourik *et al.,* 2010; Shin *et al.,* 2006; Barquero-Calvo *et al.,* 2007; Duerr *et al.,* 2009; Wolfe *et al.,* 2009).

C. jejuni possesses both types (*N*- and *O*- linked) glycosylation (Szymanski *et al*. 2003), which is present in various cellular components. Carbohydrate synthesis in *C. jejuni* has been associated with many cellular processes (Guerry and Szymanski, 2008), such as flagella assembly (Ewing *et al.,* 2009, Chaban *et al*. 2015), furthermore genes, associated with flagellin glycosylation can undergo a phase variation (Tan *et al*. 2015), which can attribute to immune evasion and affect serospecificity (Howard *et al*. 2009). The *pgl* gene (responsible for *N*-glycosylation) been associated with glycosylation of at least 45 different proteins, and has been associated with periplasmic and outer membrane proteins (Mahdavi *et al*. 2014), disruption of N-linked glycosylation in *C. jejuni* has reduced its ability to attach and invade cells *in vitro* and reduced capacity for colonisation (Szymanski *et al*. 2003). Genes responsible for *O*-linked glycosylation are adjacent to flagellin structural genes FlaA and FlaB (Guerry and Szymanski, 2008), resulting in heavy glycosylation of flagellin; up to 19 sites in each FlaA monomer decorated in *O*-linked carbohydrates figure 1.2 (Logan *et al*. 2006, Chaban *et al*. 2015) Over 40 genes have been identified, which fell into non-livestock and

live-stock associated clades, and the locus 1321-25/6 has been among these 40 genes. Gene locus 1321-25/6, has been identified to also encode for *O*-glycosylation of *C. jejuni* flagella (Howard *et al*. 2009). Removal of entire gene locus 1321-25/6, did not result in loss of motility, but has resulted in decreased autoagglutination and hydrophobicity, furthermore both entire gene locus 1321-25/6 knock out and removal of single gene from the locus (Δ1324) have resulted in low-level colonisation of avian intestine, when compare to the wild-type strain (Howard *et al.,* 2009).

Evasion of the innate immune system by *C. jejuni* is presumably a complex process, which possibly involves a number of interactions between the innate system and the bacterium on the molecular level however it has been hypothesised that locus 1321-25/6 aids the immune evasion in chicken host, which allows high level persistent colonisation. Improvement of our understanding of chicken colonisation by *C. jejuni* may be useful in vaccine development strategies, lowering the colonisation levels in chickens and improving our understanding of bacteria-host interactions further.

Figure 1.2. Flagellin glycosylation in *Campylobacter*. *C. jejuni* alters its flagellin protein structure to be unrecognizable to TLR5 (flagellin modification) or add post-translational modification to flagellins to mask target sites (glycosylation), respectively. The goal of these modifications is to modulate or avoid the host immune system to allow for a productive infection. (Chaban *et al.* 2015)



Objectives of the thesis

The main objectives of this project have been divided in to two categories: establishing a functional *in vitro* model for investigation of *C. jejuni* interaction with chicken's immune system and testing whether *O*-glycosylation of flagellin plays a role in immune evasion.

Chapter 2 – Materials and Methods

2.1. *In vitro* materials and animals

2.1.1 Cell lines

2.1.1.1 Routine maintenance and growth of COS-7 cells.

COS-7 cells were obtained from liquid nitrogen stocks, from Kaiser laboratory -80 °C storage and revived in the COS-7 growth medium (Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated foetal calf serum (Gibco), 1% L-glutamine, 1% non-essential amino acids.).

Routine maintenance of COS-7 cells involved passaging cells once confluent (at least 95%). Cells required trypsinisation in order to be split them into new flasks. Old tissue culture medium was discarded and cells were washed twice with 10 ml pre-warmed phosphate buffered saline (PBS), followed by a wash with 5 ml of trypsin/versene (1:8-1:10) solution. Trypsin/versene (5 ml) was added to cells and incubated at 37°C for 4-5 min. The flask was tapped on a bench to remove any adherent cells, and then 15 ml of complete medium was added to inhibit the activity of trypsin. Cells were pelleted by centrifugation at 1000 x *g* for 5 min. The supernatant was discarded and cells were re-suspended in 10 ml of medium, counted and seeded into T75 flasks at 7.5 x 10^5 cells/ml.

2.1.1.2. Transfection of COS-7 cells for production of recombinant chicken cytokines (IL-4, GM-CSF and CSF-1).

Transfection was performed using a DEAE/Dextran method. The day before the transfection, COS-7 cells were seeded at 6x10^6cells per T75 flask and incubated for 18-24 h at 37°C in 5%

CO_2 until 80-90% confluence is reached. Serum free DMEM (2.5 ml) was added to a bijoux, followed by addition of Endofree plasmid DNA (52 µg) and DEAE/dextran (15 µl) in that order and mixed well. COS-7 cells were then washed twice by removing media and adding PBS (5 ml), repeated twice. After the wash step DNA/DEAE complex was added to the cells and incubated for 30-60 min at 37°C in 5% CO_2, and swirled occasionally to prevent drying out of the cells. DMEM + 10% FBS (10 ml) was mixed with chloroquine (100 µl) was added to the COS-7 cells and flask returned to incubator for 2-3 h at 37°C in 5% CO_2. Following incubation, cells were washed by removing the medium and adding PBS (5 ml), followed by the addition of DMSO (10% in PBS, 10 ml) and incubated further incubation at RT for 2 min. Cell supernatant was then removed and replaced with DMEM +10% FBS and incubated or 16-24 h at 37°C in 5% CO_2. Following this incubation, the medium was removed and replaced with DMEM. If the transfection was performed for the production of recombinant cytokines, the supernatant from the transfected cells was collected after 3-5 days post media change, centrifuged at 500 x *g* for 5 min and collected into a fresh universal/falcon tube and stored at 4 °C

2.1.2 Bacterial cells

2.1.2.1. Escherichia coli.

JM109 is competent *E.coli*, purchased from Promega UK.

2.1.2.2 Campylobacter jejuni.

A number of campylobacter strains were used in this study. Wild-type strains include avian isolate strains 11168H (Jones *et al.* 2004), 81-176 and M1. Previously described mutant

strains of 11168H were used for stimulations Δ1324, Δ1321-25/6, Δ1321-25/6 repaired) (Howard *et al.* 2009).

2.1.3. Birds

Outbred lines of chickens, in order to attempt the Brown legnorn and J-line birds were used to collect tissue and bone marrow for the experiments.

2.2. *In vitro* methods

2.2.1. Culturing *Campylobacter jejuni*.

Strain confirmation

Initially wild-type and mutant strains were cultured from the glycerol stocks on CCDA plates for 48 hours under microaerophilic conditions; this was followed by selecting a single colony and re-suspending the bacterial cells in 5 ml of MH broth supplemented with Trimethoprim and cultured in the shaker (400rpm) under microaerophilic conditions for 48 hours

Campylobacter strains were cultured on CCDA plates (supplemented with Trimethoprim (5 µg/ml) and Kanamycin (15 µg/ml) (not for wild-type 11168H) for 48 h under microaerophilic conditions. A single colony of each strain was selected for a starter culture (5 ml) of MH broth supplemented with Kanamycin (not for wild-type strain of 11168H) and Trimethoprim and left in the shaker under microaerophilic conditions for 24 hours. Following incubation, starter culture (5 ml) was poured into 100ml of MH broth supplemented with Kanamycin (not for wild-type strain of 11168H) and Trimethoprim and left in the shaker under microaerophilic conditions for further 48 hours.

Fixation of *C. jejuni*

Bacterial cell were then harvested by centrifugation 2000x *g* for 10 min, the pellets were re-suspended in 4% paraformaldehyde (PFA) and left to fix for 2 hours, cells were then washed with filtered (using 0.2 µm pore size filter) 0.15 M NaCl five times (2000 x *g* for 5 min). For

control, unfixed cells, live bacterial cells were subjected to the same wash steps as fixed cells. Bacterial cells were then counted using bacteria counting kit (Invitrogen).

2.2.1.1.Bacteria counting

Bacterial cultures were serially diluted to approximate concentration of 10^6 cells/ml, and 1.0 mL volume of cell suspension was used per assay. Diluted cells were stained by adding 1 µL of SYTO® BC bacterial stain (Component A) and incubated at 37°C for 30 min, it was then mixed with microsphere standard suspension (Component B, 10 µL). The resulting mixture was used to enumerate bacterial concentration using flow cytometry analysis (FACSCalibur (BD)).

Bacterial cultures and microspheres (beads) were gated in separate gates; quantifying the signal in the microsphere gate (beads gate) provided accurate estimate of the volume analysed in the run. The microsphere standard contains 10^8 beads per mL; following the addition of the microsphere standard to the bacterial mixture, microsphere density was 10^6beads/mL and one bead represented 10^{-6} mL. The number of signal in the bacterial gate divided by the number of signal in the beads gate provided the number of bacteria per 10^{-6} of the sample.

2.2.2. Avian primary antigen presenting cells.

2.2.2.1. Collection and morphological characterisation of bone-marrow derived dendritic cells (BMDCs).

Femurs and tibias of 3-6 weeks old birds were removed and isolated from the surrounding muscle tissue using sterile instruments. Bones were placed into universals in PBS. Both ends of the bones were cut off and each bone was washed with PBS using a 10 ml syringe and 21

G needle. The cell suspension was passed through 40 µm nylon cell strainers, and then washed with PBS. Re-suspended cell suspension was overlaid over an equal amount of Histopaque 1.077 (kept at RT) and centrifuged at 1200 x *g* for 30 min (at RT) with no brakes. Cells at the interface between PBS and Histopaque 1.077 were collected and washed twice with PBS. Cells were re-suspended in PBS, counted and adjusted to a final concentration of 10^6 cells/ml. Recombinant chicken cytokines (generated as described in 2.1.1.2) were used for cell differentiation. Recombinant chicken IL-4 (1:250, - 12µl of cytokine added to each 3ml well) and GM-CSF (1:1000, -3µl of cytokine added to each 3ml well) were added to induce differentiation into DCs.

2.2.2.2 Generation and morphological appearance of bone-marrow derived macrophages (BMM).

The process followed was the same as for the dendritic cells above. Once cells were adjusted to a final concentration of 10^6 cells/ml, recombinant chicken CSF-1 (1:200) was added to induce macrophage differentiation - 5µl of cytokine added to each 1ml well

2.2.2.3. Harvesting of BMMs and BMDCs for future analysis

Primary macrophages and dendritic cells were harvested using EDTA post stimulation. Using Pasteur pipette 1 drop of 5.0M of EDTA (pH8) was added per 1 ml of media. Cells were then gently pipetted up and down to collect all the cells and were pelleted by centrifugation at 500 x *g* for 5 min for subsequent RNA isolation or flow cytometry staining procedures.

2.2.3. Nucleic acid extraction

2.2.3.1. Large scale (maxi) plasmid DNA preparation.

DNA preparation was performed using an EndoFree® Plasmid Purification kit (Qiagen) according to the manufacturer's instructions. Fresh bacterial starter cultures were streaked from glycerol stocks onto LB agar plates, supplemented with 100 µg/ml of ampicillin (LB/Amp_{100}) and cultured overnight at 37°C. On the next day, a single colony per culture was re-suspended in LB/Amp_{100} (5 ml) and cultured at 37°C for 8 h with shaking at 180 rpm to produce a seeder culture. Next, LB/Amp_{100} was inoculated with 250-500 µl of seeding culture and incubated overnight at 37°C with shaking at 180 rpm. Overnight culture was then distributed between five 50 ml Falcon tubes and cells were pelleted by centrifugation at 3000 x *g* for 60 min at 4°C. Cells were re-suspended in a lysis buffer (1 ml) and collected into one falcon tube. A second lysis buffer P2 (10 ml) was then added to cell lysates and mixed by pippeting and incubated for 20 min at RT; Neutralisation buffer P3 (10 ml) was added to the lysates and mixed by inverting until protein-genomic DNA complex is visible. The lysates were then centrifuged at 3500 x *g* for 5 min at 4 °C. Supernatant from cell lysates was then transferred to a QIAfilter Cartridge, incubated for 10 min and filtrated into new 50 ml Falcon tube. Buffer ER (2.5 ml) was added to the filtrate and incubated on ice for 30 min. Filtered lysates were then added to an equilibrated (with buffer QBT) QIAGEN-tip column, left to enter the column by gravity and then washed with buffer QC (2 x 30 ml). DNA was eluted with buffer QN (15 ml) into fresh Falcon tube and precipitated by isopropanol (10.5 ml). DNA was pelleted by centrifugation at 3000 x *g* for 60 min at 4°C. Supernatants were discarded, pellets washed with ethanol (70%, 5 ml) and centrifuged at 3000 x *g* for 10 min at 4°C. Supernatant was then discarded and any remaining liquid removed using p10 Gilson pipette, pellet were left to air dry at room temperature for 5 min and re-dissolved in endotoxin-free TE buffer (250 µl). The DNA was quantified using a Nanodrop-1000 spectrophotometer , selecting DNA-50 option in the nucleic acids section.

2.2.3.2. RNA extraction

Initial steps for RNA extraction differed, depending on the origin of the sample.

Tissue extraction from chicken pancreas

Fresh tissue was homogenised with a rotor-stator homogeniser with 600 µl of buffer RLT. The lysate was then centrifuged at full speed and the supernatant transferred to a microcentrifuge tube.

Cell culture extraction

Macrophages or DCs, were harvested by addition of EDTA 5M (pH 8.0) centrifuged at 500 x *g* for 5 min at 4 °C, supernatant discarded, RLT buffer (350 µl) added and mixed by pipetting.

Further extraction steps

Once the lysate was produced, .one volume of 70% ethanol was added to the lysate and mixed by pipetting, and 700 µl of the sample were then transferred to an RNeasy spin column which was placed in a 2 ml collection tube and centrifuged at 8,000 x *g* for 15 s. The flow-through was discarded, and this step was repeated if the sample size was greater than 700 µl. This was followed by addition of 700 µl of buffer RW1 to the RNeasy spin column, and centrifuged at 8,000 x *g* for 15 s to wash the spin column membrane. The flow-through was discarded. To wash the spin column, 500 µl of buffer RPE (containing 4 volumes of 96% ethanol) were added and centrifuged at 8,000 x *g* for 15 s. Flow-through was discarded, and this step was then repeated with 2 min centrifugation at 8,000 x *g*. The column was then placed into a fresh collection tube and centrifuged for 1 min at full speed to remove any

carryover of buffer RPE. The spin column was then placed into a 1.5 ml microcentrifuge tube and RNA was eluted with 40 µl of RNase-free water and centrifuged at 8,000 x *g* for 1 min.

2.2.4. Quantitative and non-quantitative amplification of nucleic acids

2.2.4.1.Polymerase chain reaction

Phusion High-Fidelity DNA Polymerase (Thermo Scientific, F-530S) was used to amplify the DNA, collected from *C. jejuni* cultures. PCR reaction volumes: 5x Phusion HF Buffer (10 µl), dNTP mix (dATP, dGTP, dCTP and dTTP at 10 mM, 2 µl), gene-specific forward primer (10 mM, 5 µl), gene-specific reverse primer (10mM, 5 µl), template cDNA (~50 ng, 1 µl), Phusion DNA polymerase (2 U/µl, 0.5 µl), Nuclease-free water (26.5 µl).

Cycling conditions

98	30 s	1 cycle	Initial denaturation
98	10 s		Further Denaturation
60	30 s	30 cycles	Annealing
72	90 s		Extension
72	10 min	1 cycle	Final extension

2.2.4.2. Agarose gel electrophoresis

Agarose gel electrophoresis was performed to visualise the PCR products. PCR product (5 µl) was added to 1 µl of 6x loading buffer (1x final concentration), and electrophoresed on 0.7% agarose gels at 110 V for 1.5 h. Agarose gels were prepared by dissolving 0.42 g of agarose powder in 70 ml of Tris-acetate-EDTA (TAE) buffer, and adding 7 µl of 10000x SYBR® Safe gel stain. All products were visualised using a G-Box Imager. DNA ladders (promega 1kb and 100bp; Generuler – Thermo scientific) were used to estimate product sizes.

2.2.4.3. Real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) (TaqMan assay).

Real-time qRT-PCR was used to measure the level of chicken cytokine interleukin-1β (chIL-1β) and chemokine (CXCLi2) mRNA expression in chicken DCs and macrophages. This method has been described previously (Swaggerty *et al*.,2008; Wu *et al*., 2009). Expression levels of chIL-1β and CXCLi2 mRNA were measured alongside those of a reference gene, 28S, for every sample. The 28S encodes for a ribosomal RNA component, and is constitutively expressed in all eukaryotic cells, and is therefore often used for standardisation of the target gene quantity (Swaggerty *et al*. 2008). To measure mRNA expression of 28S, chIL-1β and CXCLi2 probes and primers from Sigma were used (Table 2.1). Standard probes were labelled at the 5'end with 5- or 6- carboxyfluorescein (FAM) fluorophore, and at the 3' end with tetramethylrhodamine (TAMRA) quencher dye. The close proximity of the reporter fluorophore and the quencher allows the quencher to inhibit the fluorescence of the reporter dye (to the low level of background fluorescence). During the PCR reaction, the probe anneals to the template between the forward and reverse primers. While the template being amplified reaches the bound probe, the 5'-3' endonuclease activity of *Taq* degrades the probe, resulting in release of the reporter away from close proximity with the quencher, producing fluorescence. The level of fluorescence observed is directly proportional to the amount of PCR product in the reaction. The results were collected at the cycle threshold (C_t) value, representing the number of cycles at which fluorescence was detected above a threshold value.

A typical reaction contained FAST Universal Master Mix (5 µl), primer mix (at optimal concentration (Table 3), 0.5 µl), probe (0.25 µl), multiscribe enzyme (40x, 0.25 µl), nuclease-free water (1.5 µl), and template RNA (2.5 µl). Template RNA for test samples was diluted 1:500 for 28S and 1:5 for cytokine analysis. Standard curves were obtained from serial dilutions of mRNA from LPS-stimulated HD11 cells (previously stimulated avian macrophage cell line, which has been used as a standard; concentrations used 10^{-2}-10^{-6} for 28S and 10^{-1}-10^{-6} for chIL-1β and CXCLi2). Amplification and detection were performed using an Applied Biosystems 7500 Fast Real-Time PCR system.

Cycling conditions

48	30 min		1 cycle
95	20 s		1 cycle
95	3 s	}	40 cycles
60	30s		

To account for technical and biological variation, the C_t values for chIL-1β and CXCLi2 mRNA expression were normalised against the C_t value of the 28S RNA product for the same sample. Standard plots of C_t against $\log_{10}$(RNA) were obtained for chIL-1β, CXCLi2 and 28S RNA. Normalised C_t values were calculated using the formula $C_t+(N'_t - C'_t)*S/S'$, where N'_t is the mean C_t for 28S RNA among all samples, C'_t is the mean C_t for 28S RNA in the sample, S is the slope of the regression of the standard plot for the chIL-1β and CXCLi2 mRNA and S' is the slope of the regression of the standard plot for the 28S RNA. Results are expressed as fold change from the mRNA expression levels in the control groups.

RNA target	Probe/Primer sequence (5'-3')	Accession number	Optimal primer concentration
28S	**Probe** (FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA) **F** GGCGAAGCCAGAGGAAACT **R** GACGACCGATTTGCACGTC	X59733	0.6 mM
IL-1β	**Probe** (FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA) **F** GCTCTACATGTCGTGTGTGATGAG **R** TGTCGATGTCCCGCATGA	AJ245728	0.4 mM
CXCLi2	**Probe** (FAM)- GCCCTCCTCCTGGTTTCAG-(TAMRA) **F** TGGCACCGCAGCTCATT **R** TCTTTACCAGCGTCCTACCTTGCGACA	AJ009800	0.6 mM

Table 2.1 Taqman primers. Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) probes and primers, F- forward primer, IL-interleukin, R- reverse primer.

Chapter 3 - Results

3.1. Introduction.

Previous studies, which have focused on understanding interaction between chicken immune system and wild-type *C. jejuni* species by colonisation studies (Jones *et al.* 2004, Smith *et al.* 2008, Howard *et al.* 2009) and by using *in vitro* models and cells like chick kidney cells (CKCs), HD11 avian macrophage cell line (Smith *et al.* 2005), LHM -chicken hepatocellular carcinoma epithelial cells (Larson *et al.* 2008) or epithelian cells (Byrne et.al 2007).

Wild-type *C. jejuni* strains, which have been investigated in previous studies include "chicken specific strain" 11168H (Jones *et al*. 2004) and "human specific strain" 81-176 (Larson *et al*. 2008).

Following advances in the field of chicken immunology we can now use more appropriate *in vitro* models. Protocols are now available to generate primary bone-marrow derived dendritic cells and described by Wu *et al.* 2009; similarly, protocols of how to obtain primary bone-marrow derived macrophages have also been described (Garceau et al. 2010). This chapter describes use of these protocols to generate primary chicken antigen presenting cells(APCs): bone-marrow derived dendritic cells (BMDCs) and bone-marrow derived macrophages (BMMs) and establishing a cell culture model to investigate mechanisms by which *C. jejuni* interacts with chicken's immune.

The aim of the experiments, described in this chapter was to confirm the generation of distinguished cell populations (BMMs and BMDCs) and establish them as an *in vitro* model for investigating interations between *C. jejuni* and APCs.

3.2. Materials and methods.

BMMs and BMDCs were genenerated and cultured,bone marrow collected from same birds was pooled together (2.2.2.1 and 2.2.2.2), both BMMs and BMDCs were cultured for six days prior to stimulation. Bacterial culture of *C.jejuni* was cultured as described previously (2.2.1) and bacterial numbers were adjusted using bacteria counting kit as described in (2.2.1.1). Post-stimulation, both BMMs and BMDCs were harvested using EDTA (2.2.2.3), RNA extracted (2.2.3.2) and analysed for expression of chIL-1β using Taqman qRT-PCR (2.2.4.3).

3.3. Results

Morphological appearace of dendritic cells.

Primary cells were cultured for 6 days in the presence of recombinant chicken IL-4 and GM-CSF (41°C, 5% CO_2). After 6 days, cells were viewed under the light microscope (Figure 3.1), cells have developed typical morphological appearance - have irregular shape, some are detached and are forming clumps of cells on top of the monolayer, as previously described in Wu *et al.,* 2009.

Morphological appearance of macrophages

Primary cells, cultured for 6 days in the presence of the CSF-1 (41°C, 5% CO_2) were viewed under a light microscope. Appearance of cells observed - “fried egg” appearance with extensive dendrites and round cell aggregates on top (Figure 3.2), which is confirmed to be a typical morphology, described previously for avian macrophages (Garceau *et al*. 2010).

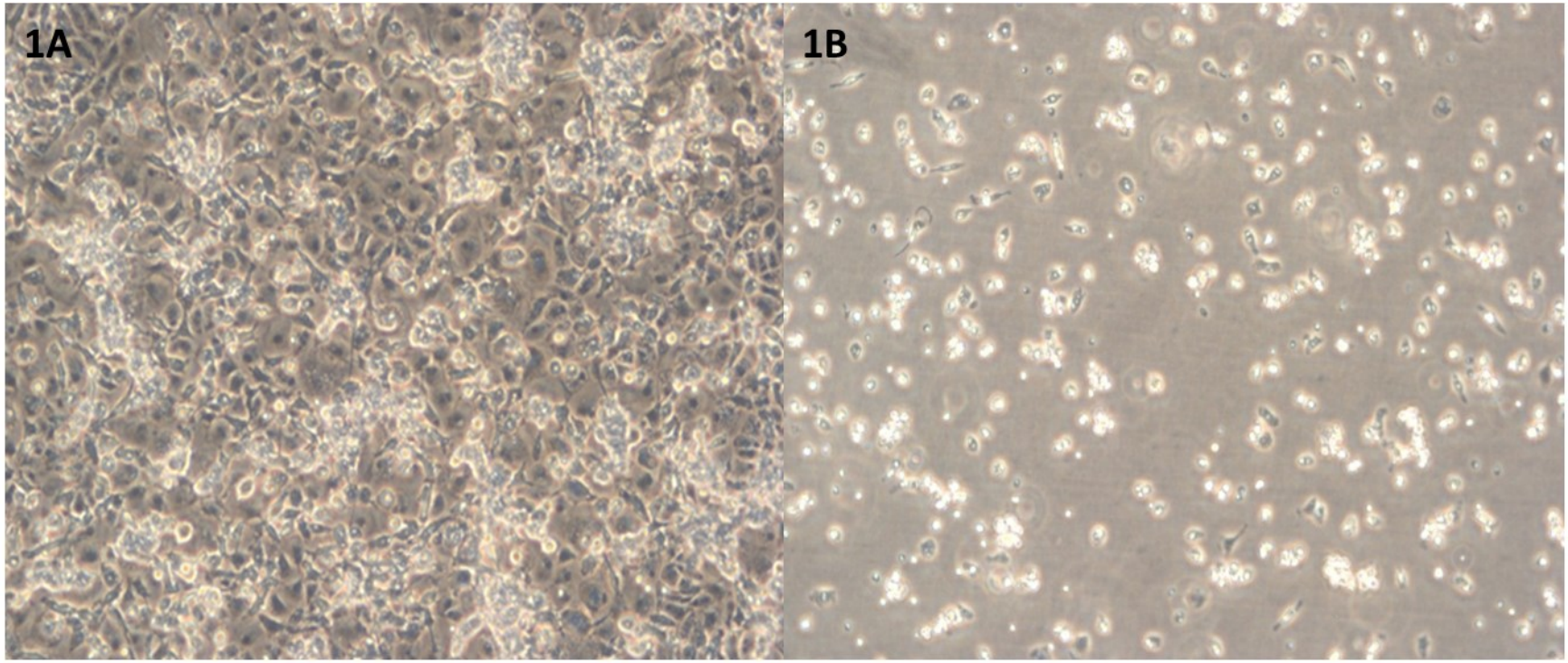


Figure 3.1. Bone marrow-derived dendritic cells (BMDCs) seeded at 10^6 cells per ml and incubated for 6 days. 1A. Cells were supplemented with recombinant chicken GM-CSF (1:250) and IL-4 (1:250) 1B. Control group; cells were not supplemented with cytokines.

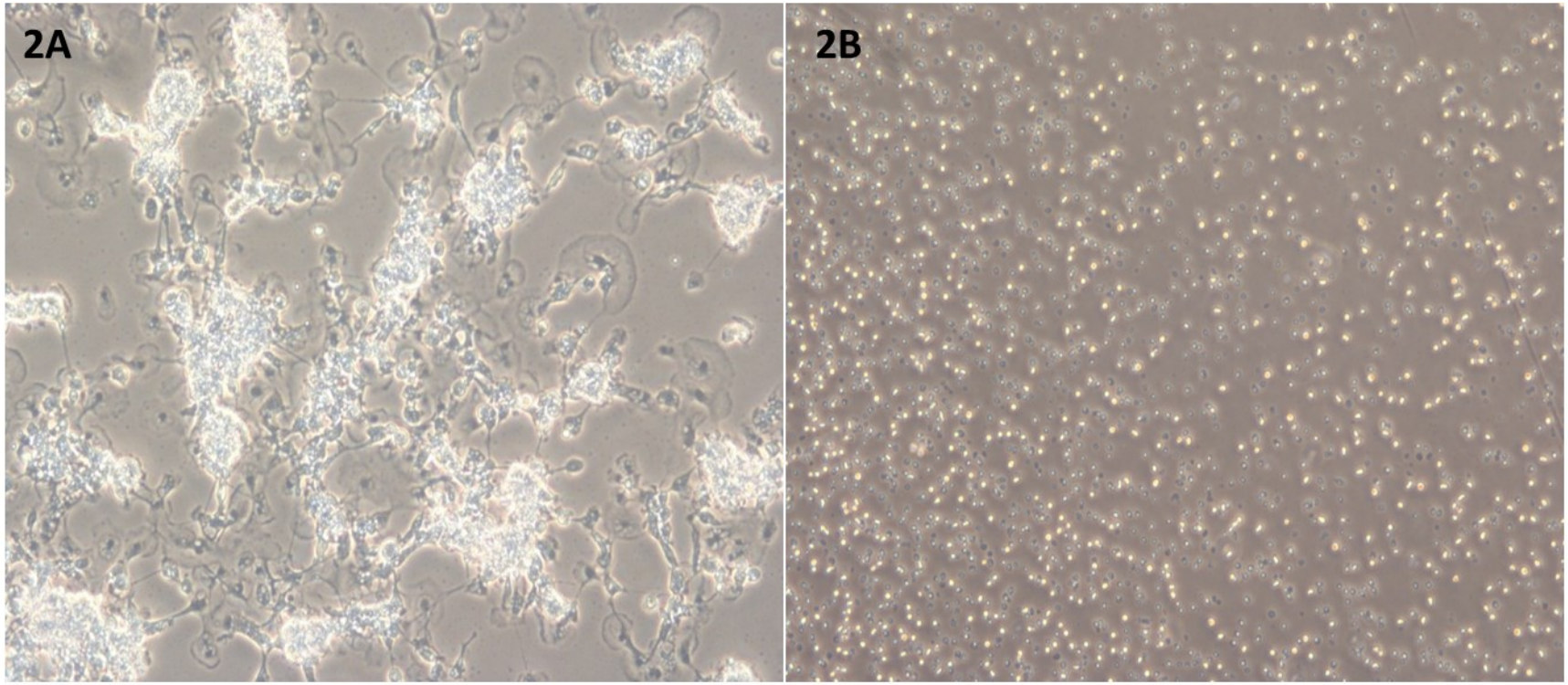


Figure 3.2. Bone marrow-derived macrophages (BMMs) seeded at 10^6 cells per ml and incubated for 6 days. (A) Cells were supplemented with recombinant chicken CSF-1 (1:200). (B) Control group; cells were not supplemented with cytokines.

Stimulations of BMMs and BMDCs with different concentrations of Lipopolysaccharide (LPS).

After 6 days of growth, cells were stimulated with LPS in order to establish optimum concentration for use as a positive control. Cells were stimulated with different concentrations of LPS (50 ng/ml – 400 ng/ml) for 2, 4 or 6 hours for macrophages and 2 or 4 hours for dendritic cells (at 41°C, 5% CO_2). Cells were harvested post stimulation using EDTA (2.2.2.3), mRNA was extracted (2.2.3.2), and analysed for expression of chIL-1β cytokine using Taqman qRT-PCR (2.2.4.3).

The mRNA expression of chIL-1β in BMMs and BMDCs in response to LPS stimulations was higher for both BMDCs and BMMs, when compared to un-stimulated control (Figure 3.3). It was however evident that this up-regulation of chIL-1β mRNA expression in these experiments was not dose dependent, as it did not increase as the LPS dose increased.

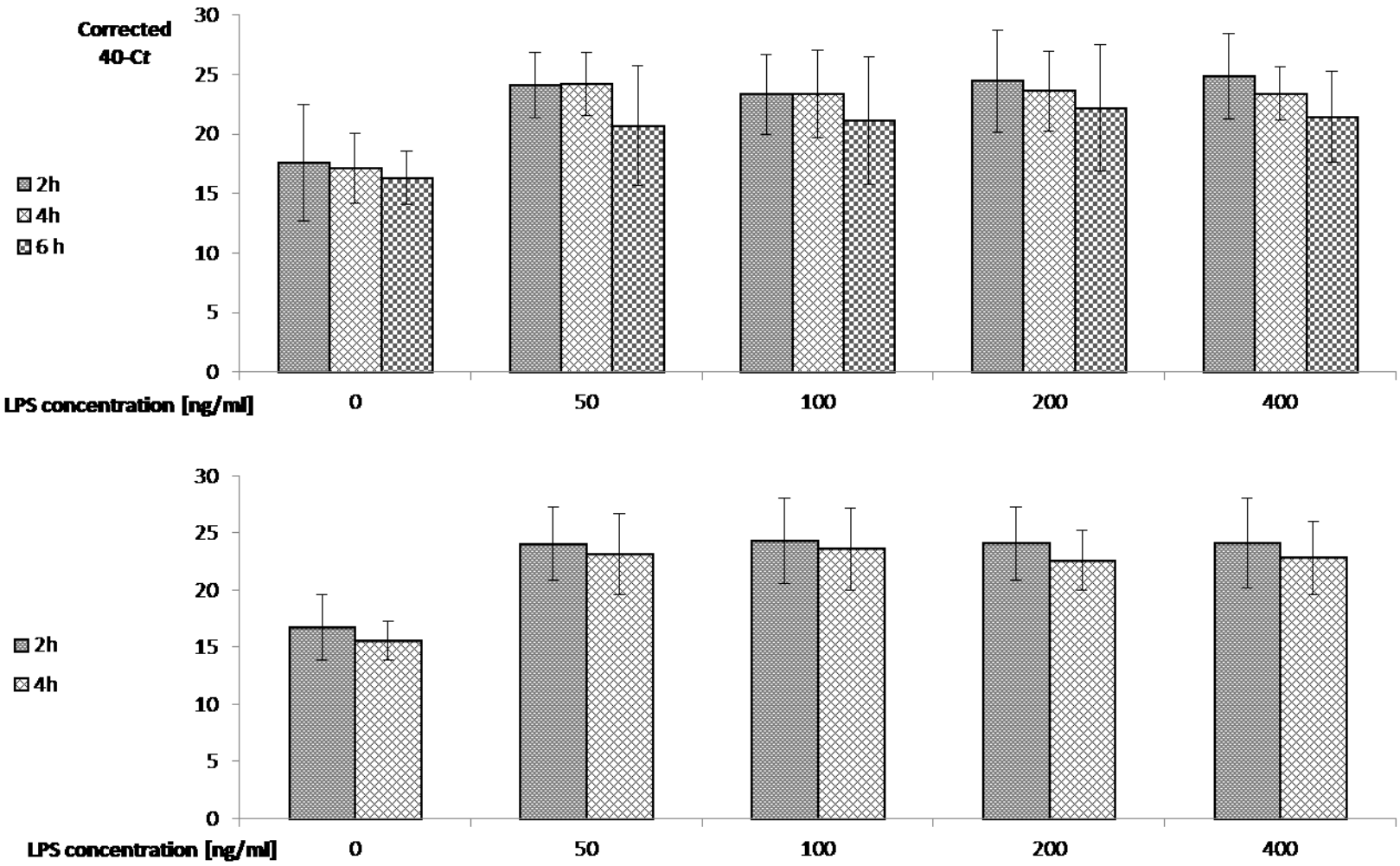


Figure 3.3. Expression levels of IL-1β mRNA in response to stimulations with LPS in (A) BMMs and (B) BMDCs ***stimulated with range of concentrations of LPS (0, 50, 100, 200 and 400 ng/ml) for 2,4 and 6 (2 and 4 hours for BMDCs. Data are presented as the average corrected 40-Ct values of two individual birds ±SD.***

Stimulations of primary BMMs and BMDCs with various *C. jejuni* strains and concentrations.

BMDCs and BMMs were generated and cultured as described previously, bone marrow samples were collected from six different birds, bone marrow from same birds was pooled together (2.2.2.1 and 2.2.2.2 respectively). Following six days of culture, BMMs and BMDCs were stimulated with wild-type *C. jejuni* strains: M1, 81-176 and 11168H at 10^7 and 10^8 bacteria per ml for 4 hours at 41°C, 5% CO_2. Cells were harvested post stimulation using EDTA (see section 2.2.2.3); mRNA was extracted from BMDCs and BMMs as described in section 2.2.3.2. Collected mRNA samples were analysed for expression of chIL-1β and CXCLi2 cytokines using Taqman qRT-PCR (as described in section 2.2.4.3).

The mRNA expression of chIL-1β from BMMs, stimulated with *C. jejuni* wild-type strains 81-176 and 11168H was increased, in comparison with a non-stimulated control, there was no apparent increase in chIL-1β expression in response to stimulation with M1 wild-type strain (Figure 3.4). Observed mRNA expression levels of CXCLi2 have expressed similar pattern of chIL-1β.

The mRNA expression of chIL-1β and CXCLi2 from BMDCs, stimulated with *C. jejuni* wild-type strains 81-176, M1 and 11168H increased, when compared to non-stimulated control. In this experiment, observed results suggest that mRNA expression not affected by *C. jejuni* strains (Figure 3.5).

Figure 3.4. Expression levels of IL-1β mRNA (A) and CXCLi2 mRNA(B) as measured by qRT-PCR. *In primary chicken BMMs in response to stimulations with media (U), LPS 100 ng/ml (L), and different C. jejuni wild-type strains: M1 at 10^7 bacteria per ml (10^7) and 10^8 bacteria per ml (10^8), 81-176 at 10^7 bacteria per ml (10^7) and 10^8 bacteria per ml (10^8), and 1168H at 10^7 bacteria/ ml (10^7) and 10^8 bacteria per ml (10^8). Data are expressed as corrected 40-Ct ±SEM, n=6.*

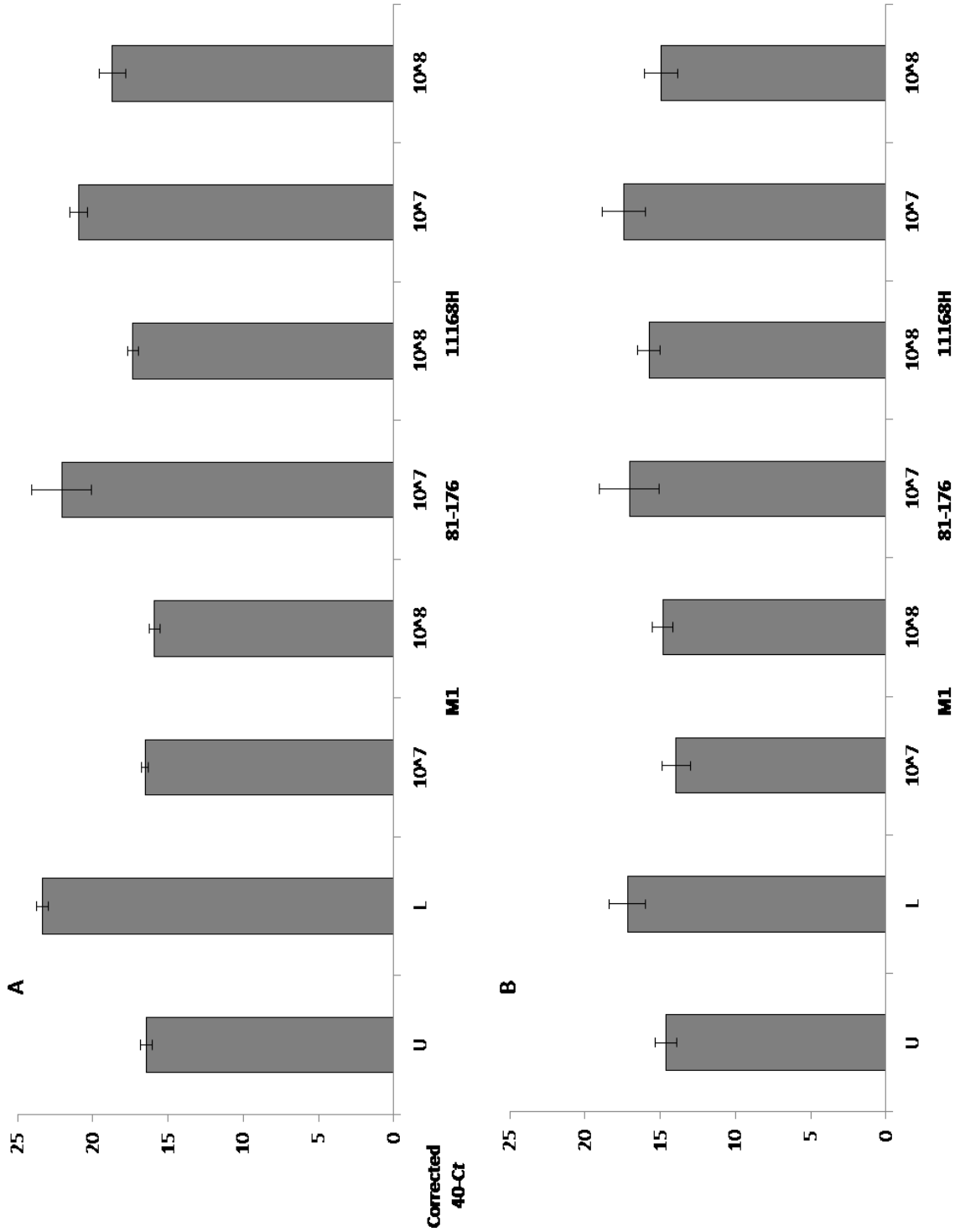
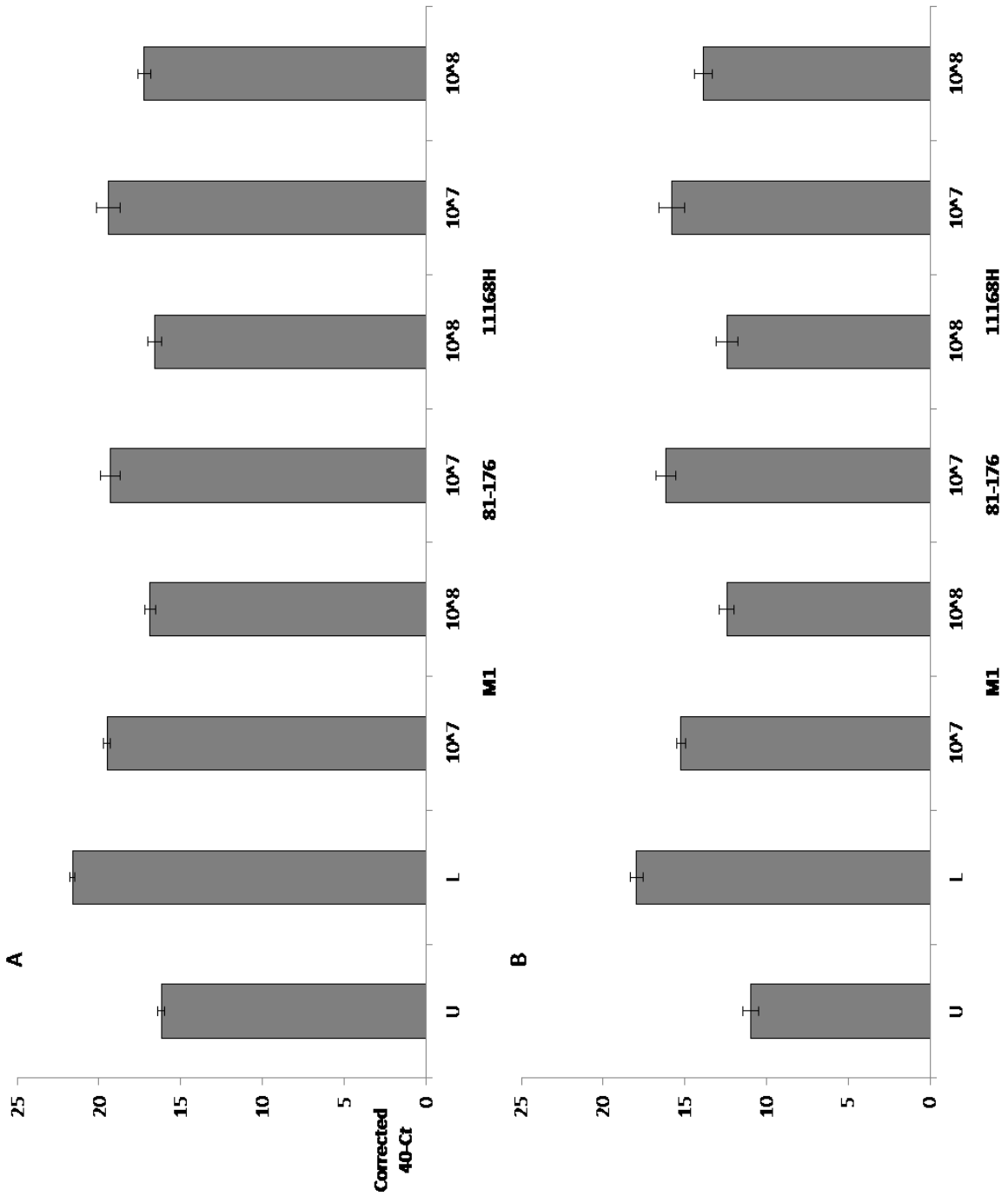


Figure 3.5. Expression levels of IL-1β mRNA (A) and CXCLi2 mRNA(B) as measured by qRT-PCR. *In primary chicken BMDCs in response to stimulations with media (U), LPS 100 ng/ml (L), and different C. jejuni wild-type strains: M1 at 10^7 bacteria per ml (10^7) and 10^8 bacteria per ml (10^8), 81-176 at 10^7 bacteria per ml (10^7) and 10^8 bacteria per ml (10^8), and 11168H at 107 bacteria per ml (10^7) and 10^8 bacteria per ml (10^8). Data are expressed as corrected 40-Ct ±SEM, n=6.*



The mRNA expression levels of chIL-1β in primary BMMs and BMDCs, simulated with two different batches of same *C. jejuni* strain.

BMM and BMDCs were cultured as previously described (2.2.2.1 and 2.2.2.2 respectively). Two different batches of *C. jejuni* were cultured and fixed in PFA as previously described; and bacterial numbers were established, and bacterial numbers were adjusted to 10^8 bacteria per ml, and then cells were stimulated with *C. jejuni* wild-type strain 11168H at 10^8 bacteria per ml for 4 hours at 41°C, 5% CO_2. BMMs and BMDCs were harvested (2.2.2.3), RNA extracted (2.2.3.2) and analysed for expression of chIL-1β using Taqman qRT-PCR (2.2.4.3).There was no apparent difference between mRNA expression levels of chIL-1β in response to different batches (data not shown).

Comparison of mRNA expression levels of chIL-1β in primary BMMs and BMDCs, simulated with live and fixed and fixed *C.jejuni*.

BMM and BMDCs were cultured as previously described (2.2.2.1 and 2.2.2.2 respectively). Same batch of *C. jejuni* was cultured as previously described; and fixed in PFA as previously described; and bacterial numbers were established, and bacterial numbers were adjusted to 10^8 bacteria per ml, and then cells were stimulated with *C. jejuni* wild-type strain 11168H at 10^8 bacteria per ml for 4 hours at 41°C, 5% CO_2. There was no notable difference in mRNA expression levels of IL-1β in response to bacteria fixed in PFA 4% and non-fixed bacteria (data not shown).

3.4. Discussion

In this chapter we have used current tissue culture models in an attempt to mimic the *in vivo* interactions between innate chicken cells and bacterial cells, and have optimised the experimental procedures in order to establish a functional *in vitro* model for further understanding of bacteria-host interaction in chickens.

Primary BMDCs and BMMs were cultured as previously described, their morphological appearance resembled the published description (Garceau et al. 2010, Wu *et al.* 2009). BMDCs and BMMs were then stimulated with lipopolysaccharide (LPS), in our experiments there was no noticable difference in mRNA expression levels of chIL-1β in response to different concentrations of LPS (50, 100, 200 and 400 ng/ml), in previous studies LPS concentration used was 200ng/ml (Wu *et al.,* 2009). Using previous literature and observed results we have concluded that use of 100ng/ml was acceptable in future experiments.

Use of fixed bacteria when compared to use of non-fixed bacteria of the same strain on APCs did not affect the mRNA expression of chIL-1β and, therefore used in future experiments.

Different batches of the same strain of *C. jejuni* did not result in different expression of chIL-1β. These results have confurmed that use of different batches was indded acceptable for future experiments.

Wild-type strains of *C. jejuni* have generally displayed an increase in production of of chIL-1β and CXCLi2, however the 11168H strain was chosen for future exeriments as it is a parent strain for mutant strains used in the future experiments.

3.5. Conclusion

This experimental chapter provided adequate preliminary information which is used to set up the main experiments as described in the next chapter. Phenotypes of both BMDCs and similar to previously described.

Positive controls of LPS 100ng/ml and wild-type *C. jejuni* 11168H were established for future experiments.

Chapter 4 - Results

4.1. Introduction

C. jejuni is a frequent coloniser of chickens gastrointestinal tract and is able to colonise in hight numbers, producing a limited immune response (Smith *et al*. 2008). *Campylobacter jejuni* strain 11168H has been shown to colonise chickens efficiently in previous studies (Jones et al. 2004). A cluster of genes 1321-25/6 was found to this cluster of genes was prevalent in live-stock campylobacter isolates, especially chickens (Champion *et al*. 2005), these genes are responsible for *O*-linked glycosylation of flagellin in *C. jejuni*.

Glycosylation has been previously shown to be an important factor for colonisation ability of *C. jejuni* (Jones *et al.* 2004). It has also been illustrated, that mutant strains Δ1324 and Δ1321-5/6 were not able to colonise chickens as efficiently as wild-type strain. It has be hypothesised that more efficient immune response against mutants with altered ability for *O*-glycosylation might be due to a more efficient immune response being mounted against *C. jejuni* (Howard et al. 2009).

In this chapter we have attempted to investigate this hypothesis by stimulating chicken BMDCs and BMMs with wild-type 11168H and mutant strains derived from it: Δ*flaA* – mutant strain completely lacking flagella (Jones *et al.* 2004); Δ1321-25/6 and Δ1324, flagellin *O*-glycosylation cluster and a single gene from this cluster (Howard et al. 2009).

4.2. Materials and Methods

To investigate the responses of chicken antigen presenting cells to *Campylobacter jejuni*, BMDC and BMM were cultured from J-line birds as described previously (2.2.2.1 and 2.2.2.2 respectively).

BMDCs and BMMs were stimulated with media, 100ng/ml of LPS, and 10^8 bacteria per ml (enumerated as described previously 2.2.1.1) of *C. jejuni* 11168H and it's mutant strains (Δ1234, Δ1321-25/6 (and it's repair mutant), ΔFlaA) for 4 hours at 41°C, 5% CO_2.

To reduce technical variation each stimulation assay contained each own control cells, which included unstimulated (negative control), stimulated with 100 ng/ml of LPS (positive control) and wild-type *C. jejuni* (11168H strain positive control).

Post-stimulation, RNA from BMDCs and BMMs was collected (2.2.2.3 and 2.2.3.2) and expression levels of chIL-1β were measured using Taqman qRT-PCR (2.2.4.3). Data were then presented as corrected 40-C*t* and statistically analysed for significant differences between treatments using paired t-test. Five different birds were used to bone marrow for generation of BMMs and four different birds were used to bone marrow for generation of BMDCs for these experiments.

4.3. Results

Changes in mRNA expression levels of chIL-1β in avian APCs in response to stimulation with *cj*Δ1321-1325/6.

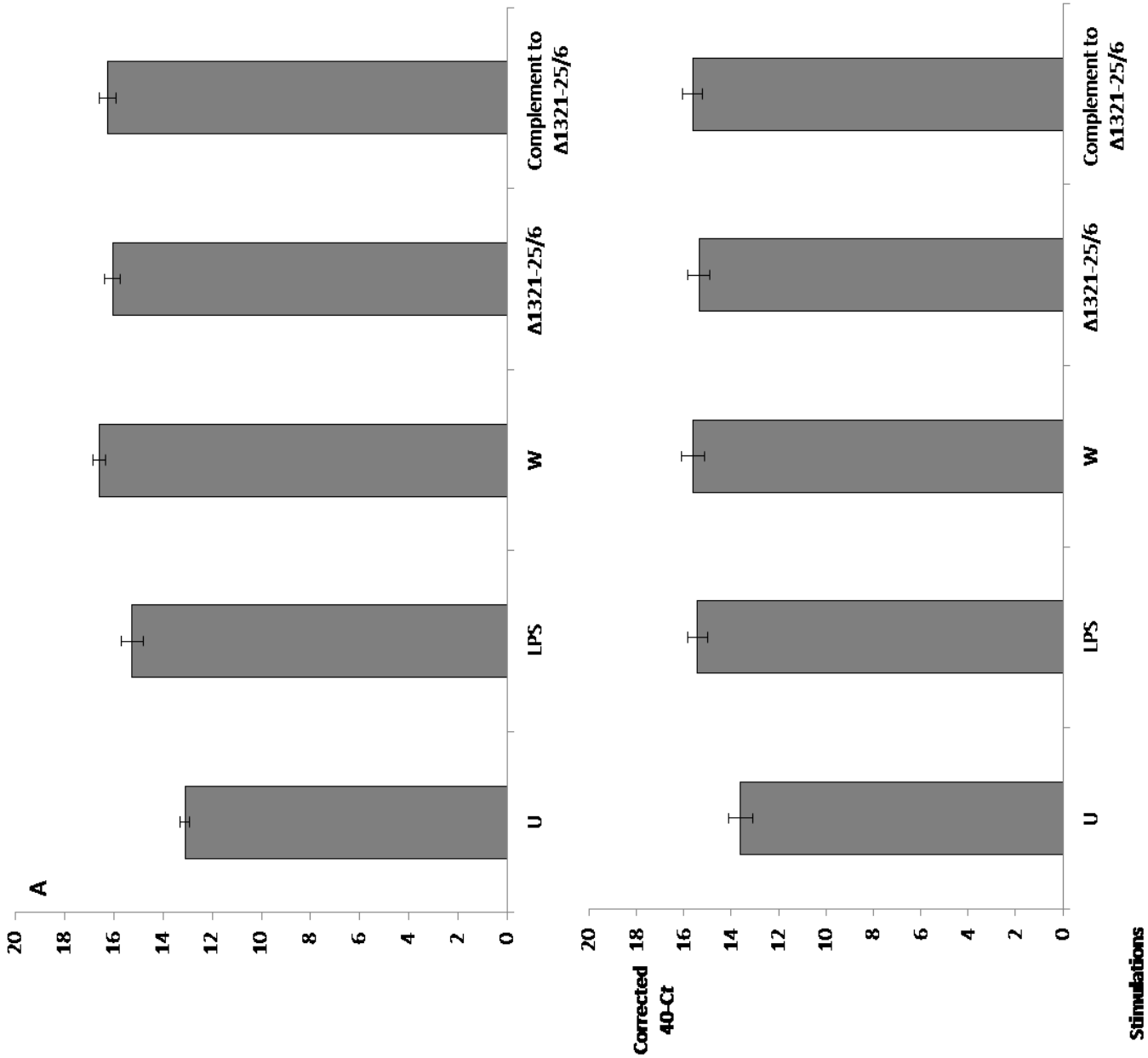
Expression levels of chIL-1β mRNA in primary chicken BMDCs and BMMs, following stimulation with knock out mutant of the Δ1321-1325/6 locus (Howard et al., 2009) and a repaired strain of Δ1321-1325/6 locus are summarised in the figure 6.

BMMs stimulated with Δ1321-25/6 mutant strain have shown a significant decrease ($p=0.042$) when compared with the mRNA expression levels of chIL-1β in BMDCs, stimulated the wild-type *C. jejuni* control (11168H). The chIL-1β mRNA expression levels were increased when BMMs were stimulated with repair mutant of Δ1321-25/6, but is not statistically significant ($p=0.139$), when compared to mRNA expression of chIL-1β observed in response to mutant strain Δ1321-25/6 stimulation figure 6.

BMDCs, stimulated with Δ1321-25/6 mutant strain have displayed a tendency for decreased mRNA expression levels of chIL-1β ($p=0.052$) when compared with the mRNA expression levels of chIL-1β in BMDCs, stimulated the wild-type *C. jejuni* control (11168H). The mRNA expression levels of chIL-1β in BMDCs, stimulated with a repair strain of Δ1321-25/6 was restored to levels, similar to ones observed in stimulation with wild-type *C. jejuni* strain (11168H), however it was not statistically significant ($p=0.626$) figure 4.1.

Figure (4.1) IL-1β mRNA expression in unstimulated and stimulated BMDCs (A) and BMMs (B), as measured by qRT-PCR.

IL-1β mRNA expression levels were analysed in Unstimulated (U) or stimulated with 100ng/ml of Lipopolysaccharide (LPS), (W) wild-type strain of Campylobacter jejuni 11168H, (Δ1321-1325/6) mutant strain of Campylobacter jejuni with knock out of Δ1321-1325/6 locus, and (complement Δ1321-1325/6) repair mutant of Δ1321-1325/6 knock out. for 4 hours. Data are presented as the average corrected 40-Ct values of five (BMM) and four (BMDC) individual birds ±SEM.



Changes in mRNA expression levels of chIL-1β in avian APCs in response to stimulation with *C. jejuni* ΔFlaA mutant strain

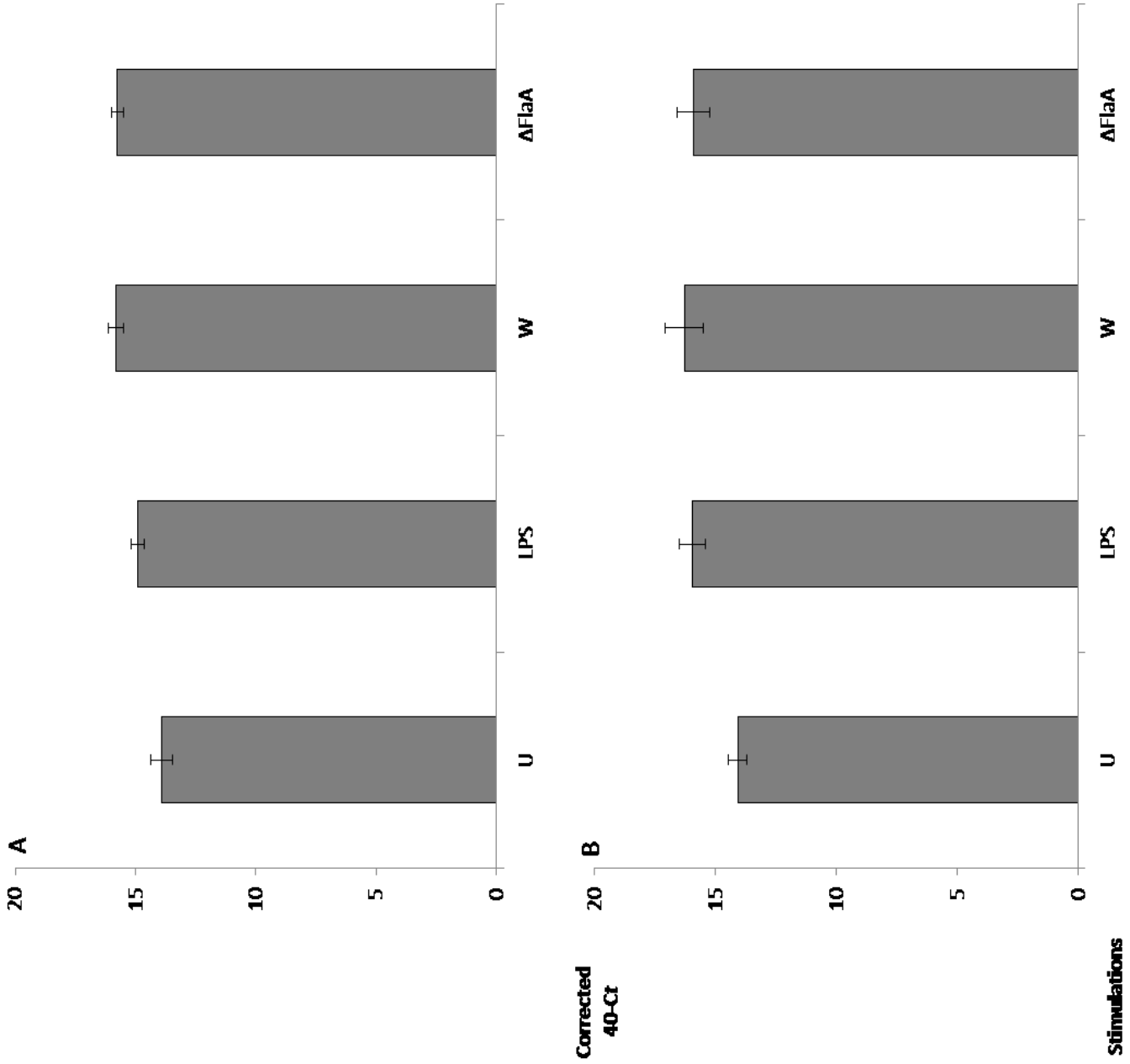
Changes in mRNA expression levels in BMMs and BMDCs in response to smilulation with knock out mutant of the flagellin component A – Δ*fla*A (Jones *et al*. 2004) to are summarised in the figure 4.2.

BMDCs, stimulated with with *C. jejuni* knock out mutant of the flagellin component A – Δ*fla*A, have not displayed any significant decrease (p=0.552) in mRNA expression of chIL-1β when compared with the mRNA expression levels of chIL-1β in BMDCs, stimulated the wild-type *C. jejuni* control (11168H) (figure 4.2).

Similarly BMMs stimulated with *C. jejuni* knock out mutant of the flagellin component A – Δ*fla*A, have not displayed any significant decrease (p=0.503) in mRNA expression of chIL-1β when compared to the mRNA expression levels of chIL-1β in BMMs, simulated with the wild-type *C. jejuni* control (11168H) (figure 4.2).

Figure (4.2) IL-1β mRNA expression in unstimulated and stimulated BMDCs (A) and BMMs (B), as measured by qRT-PCR.

IL-16 mRNA expression levels were analysed in Unstimulated (U) or stimulated with (LPS) 100ng/ml of Lipopolysaccharide, (W) wild-type strain of Campylobacter jejuni 11168H, (ΔFlaA) mutant strain of Campylobacter jejuni with knock out of FlaA for 4 hours. Data are presented as the average corrected 40-Ct values of five individual birds ±SEM.



Changes in mRNA expression levels of chIL-1β in avian APCs in response to stimulation with *cj*Δ1324

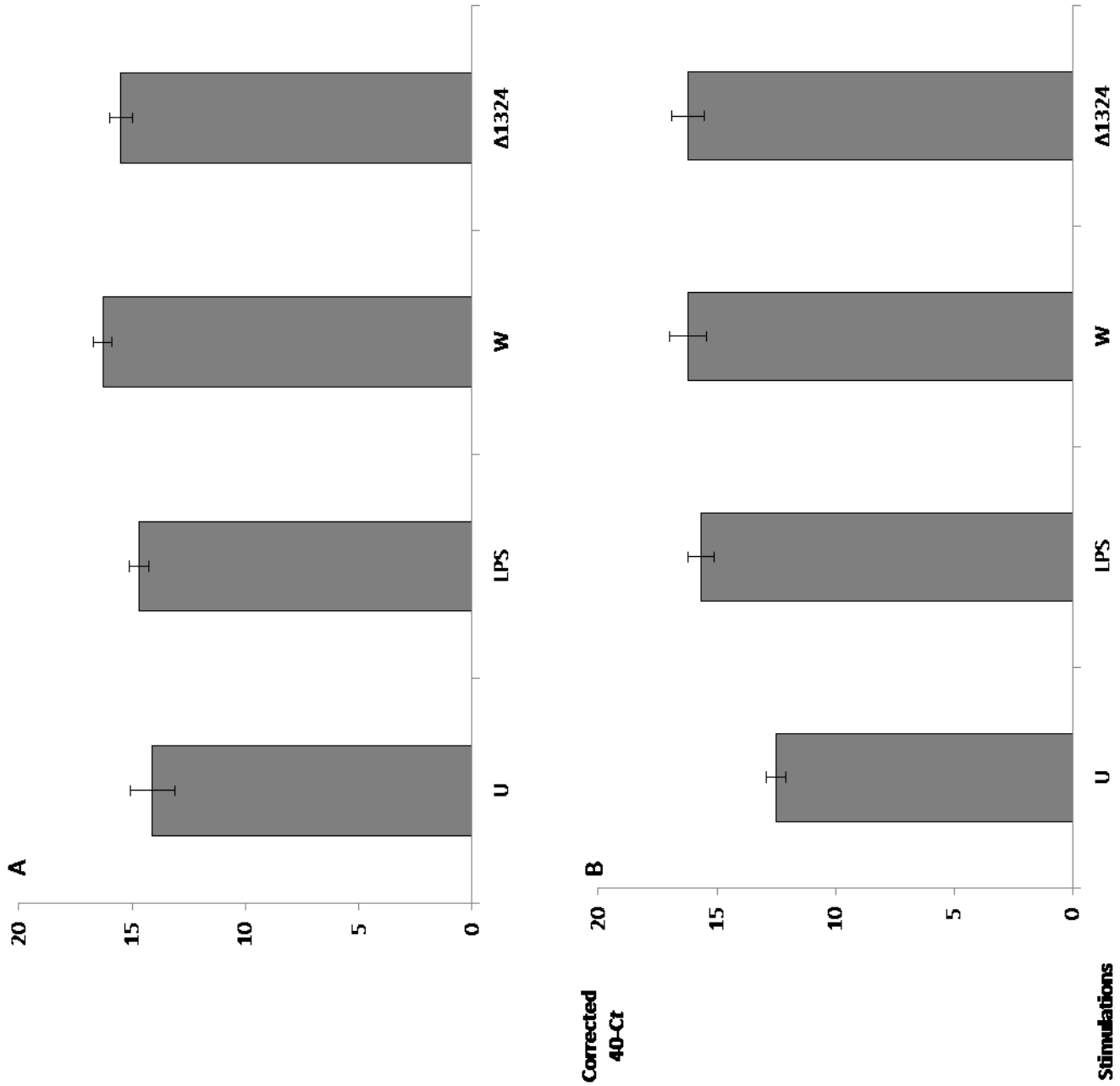
Changes in mRNA expression of chIL-1β in primary chicken BMDCs and BMMs, in response to stimulation with *C. jejuni* knock out mutant Δ1324 (Howard *et al.* 2009) are summarised in the figure 4.3.

Stimulation of BMMs with Δ1324 mutant strain of *C. jejuni* has resulted in non-significant decrease (p=0.989) in mRNA expression of chIL-1β, when compared to the mRNA expression levels of chIL-1β in BMMs, simulated with the wild-type *C. jejuni* control (11168H) figure 4.3.

The expression levels of chIL-1β mRNA levels in BMDCs were significantly decreases (p=0.013), when compared to the mRNA levels of chIL-1β in BMDCs, when compared to the mRNA expression levels of chIL-1β in BMDCs, simulated with the wild-type *C. jejuni* control (11168H) figure 4.3.

Figure (4.3) IL-1β mRNA expression in unstimulated and stimulated BMDCs (A) and BMMs (B), as measured by qRT-PCR.

IL-1β mRNA expression levels were analysed in Unstimulated (U) or stimulated with (LPS) 100ng/ml of Lipopolysaccharide, (W) wild-type strain of Campylobacter jejuni 11168H, (Δ1324) mutant strain of Campylobacter jejuni with knock out of 1324 gene, for 4 hours. Data are presented as the average corrected 40-Ct values of five (BMM) and four (BMDC) individual birds ±SEM.



4.4. Discussion

Stimulation of BMMs and BMDCs with ΔFlaA mutant strain did not result in a significant decrease in mRNA expression of chIL-1β, interestingly this mutant strain has also failed to colonise chickens 7 days post infection (Jones *et al.* 2004) This could be indicative that lack of flagellin assembly might not be an important factor for production of chIL-1β mRNA in avian APCs. Flagellin mutants have been shown to reduce their colonisation ability in chickens. The use of ΔFla A mutant strain was interesting as both Δ1324 and Δ1321-25/6 are genes responsible for flagellin, these data suggest that observed changes in mRNA expression levels of chIL-1β are not triggered by flagelin interaction with APCs. Interestingly, *C. jejuni* was also not able to activate NF-κB via chicken TLR5 (de Zoete *et al*. 2010).

Expression levels of chIL-1β mRNA in BMDCs was significantly decreased when stimulated with mutant strain Δ1324, in comparison with mRNA levels of chIL-1β observed in to the wild-type stimulation.

Decrease in mRNA of chIL-1β was not significant in BMMs stimulated with with mutant strain *cj*Δ1324, compared to the wild-type, however there has been a significant decrease in mRNA expression levels of chIL-1β, stimulated with the same strain (*cj*Δ1324), when compared to BMDCs stimulated with wild-type *C. jejuni*. It is unclear what causes the difference in responses between BMDCs and BMMs.

Significant decrease in IL-1β mRNA has been obsereved in BMMs post stimulation with *cj*Δ1321-25/6 when compared to the wild-type. Notable, but not statistically significant decrease in IL-1β mRNA has been obsereved in BMDCs post stimulation with *cj*Δ1321-25/6 when compared to the wild-type. Interestingly, both BMDCs and BMMs have displayed a

decreased mRNA expression levels of chIL-1β in response to *cj*Δ1321-25/6, in this case the lack of statistical significance, observd in BMDCs may be attributed to the low number of repeats.

Chapter 6 - Discussion

Rationale of the work and Importance of this project on the global scale.

C. jejuni is a major cause of bacterial gastroenteritis in the developed world, furthermore, there are serious complications associated with *C. jejuni* infections in the form of neuropathies Guillain-Barré syndrome and Miller Fisher syndrome (Ang *et al.* 2002)

Understanding of initial campylobacter colonisation, is important, as it may be an important factor requied for establishment of high colonisation levels of *C. jejuni* in chickens. Reducing colonisation numbers in chickens would increase effectiveness of farm biosecurity measures, which would, in turn reduce the reservoir for human infection (Kaiser, 2010; Huyghebaert et al., 2010).

The immune response is a complicated process; it is vast and involves many outputs from both innate and adaptive immune cells. In this project we have attempted to elucidate the mechanisms involved in initial recognition of the *Campylobacter jejuni* by the macrophages and dendritic cells, and factors, which might be relevant for the persistent *C. jejuni* colonisation in chickens. Primary avian BMDCs and BMMs were chosen as they were previously described and were easily reproducible, furthermore both did not involve large numbers of experimental animals (unlike for heterophil generation). Both macrophages and dendritic cells are involved in antigen presentation and are often first ones to be in contact with pathogen. The activation of innate immune response will in turn lead to the heterophil infiltration of the site of the infection (Smith *et al*. 2009). **Justification of concentrations, time points and strains.**

As in previous research published by Smith *et al*. 2005, we have used points for the initial immune response to LPS at 2, 4 and 6 hours. However, in order for most consistent response to *C. jejuni* and positive control (LPS) has been observed at 4 hours post stimulation.

Using the parafolmaldehyde (PFA) to fix bacterial cells prior to the stimulation has enabled us to ensure that bacterial population does not change in the course of the experiment additionally changes in the oxygen concentrations would have altered the "state" (coccoid appearance) of the bacterial cells (Smith *et al.* 2016, Oh *et al*. 2015). Concentration selection was determined by producing serial dilutions of *C. jejuni* suspension and stimulating for 4 hours. Biological significance of these observations however has not been confirmed as, avian macrophages or dendritic cells have not been described to be in contact with such high concentration of bacterial cells per an immune cell.

We have investigated the response to different wild-type strains of *C. jejuni* to illustrate the difference in the response produced. Ability of different strains of *C. jejuni* to elicit a varied immune responses avian APCs, is in accordance with previous studies (Humphrey *et al*. 2014). The effect produced by APCs in response to different wild-type strains was not pursued further as wilt-type strain 11168H is a parent strain to all of the mutant strain we have used and was used as a wild-type control in the subsequent experiments.

APC response to stimulation with mutant *C. jejuni* strain ΔFlaA.

Both BMMs and BMDCs have not displayed a decrease in mRNA expression of chIL-1β in response to flagellin knock out mutant ΔFlaA, it is hypothesised that flagellin per se may not be important in the immune detection of *Campylobacter* by chicken APCs, unlike in the

bacterial species, Campylobacter flagellin does not appear to be highly immunogenic and has been described to not be by both chicken and human TLR5 (de Zoete et al. 2010), and has lack of binding sites for hTLR5 (Guerry and Szymanski, 2008). Similarly *Helicobacter pylori* flagellin proteins are not detected by TLRs in mammals (Amieva and El-Omar 2008). However the flagellin cap protein *FliD* have been demonstrated to be important for cell binding in mammalian cells (Freitag *et al.* 2017).

APC response to stimulation with mutant *C. jejuni* strain Δ1321-25/6 and Δ1324

Both BMMs and BMDCs have displayed a decrease in chIL-1β production in response to stimulations with both Δ1321-25/6 and Δ1324, both of these mutant strains have displayed a reduced ability to colonise avian gut, resulting in low level persistence, unlike the wild-type strain (Howard *et al.* 2009). Similarly, altering the flagellin *O*-glycosylation in *Pseudamonas* species has resulted in decrease in production of human IL-8, when compared to stimulation with wild-type strains (Verma *et al.* 2005).

The biological relevance of reduced chIL-1β production might lay in the function of glycosylation itself. Glycosylation might not only be important in masking the bacterial surface to avoid detection, it has also been demonstrated in an initiation of immune response (Tan *et al.* 2015), it is possible that locus 1321-1325/6 of *C. jejuni*, is important in initial interaction with immune system, which leads into raising a response, which is not sufficient for clearance of *C. jejuni* which may enable high-level persistent colonisation *Salmonella* (Tsolis *et al.* 2008) and human pathogen *H. pylori* can elicit both proinflammatory and immunosuppressive effects in human stomach, it is hypothesised that it induces a robust but specific form of chronic inflammation that is ineffective in clearing the infection, while avoiding forms of inflammation that would eliminate it (Amieva and El–

Omar 2008). It is possible that this strategy was initially adapted by now commensal bacteria, which are inducing low level inflammation in the gut, yet are not cleared, and can rapidly spread, human gut commensals are able to colonise in high numbers and persist for long periods of time. The glycosylation of *Bactroides spp* is also important for colonisation ability for its biological niche (Fletcher *et al.* 2009).

Pro-biotic bacteria in human gut (Thomas and Versalovic 2010) decreased IL-1β and other pro-inflammatory cytokines by modulating the signalling pathways such as NF-κB in gut epithelial cells, as well as promote survival and musin secretion and factors to help induction of "gut homeostasis". *C. jejuni* seems to be initiating a long term inflammation in the guts of the chicken; interestingly the birds, which were more successful at clearance of the *C. jejuni* have displayed an induction of regulatory (anti-inflammatory) responses, such as IL-10 and Tregs (Humphrey et al. 2014).

Previous studies of *Salmonella* spp have confirmed that altering glycosylation the pathogen and makes it more "detectable" by intestinal epithelial cells, wild-type strain have display in activation of intestinal epithelial cells, but not macrophages (Durr *et al*. 2009).

For example inflammation leads to vasodilatation and may result in "leaky gut" which will increase the nutrient supply; it may also assist in elimination of competitive species. This suggests that *C. jejuni* has adapted to avian immune system and is able to manipulate to avoid the clearance, resulting in high levels of persistence. The main aim of pathogen is to survive, so inducing devastating immune response which, will result in the death or serious impairment of the host will not benefit the bacteria, instead manipulating the immune response, in order to allow high level, persistent colonisation and rapid spread, results in the conservation of the bacterial species.

Limitations

Limitations of this model and what could have been improved, for example use of experimental birds which are specific-pathogen free (SPF), non-vaccinated inbred line birds, that share same MHC as in studies by (Smith *et al*. 2008 Howard *et al*. 2009, Wu *et al.* 2009, Jones *et al.* 2004), would have possibly resulted in more universal response, therefore producing more clear data as the data we have collected had high standard deviation both between positive control (LPS) and bacterial stimulations. Some birds have been producing higher fold of mRNA, other much smaller.

There is evidence that reactive nitrogen species also contribute to the clearance process of *C. jejuni* in human host (Iovine *et al.* 2008) similarly iNOS production was induced in CKC and HD11 cells (Smith *et al.* 2005). It has also been identified that *C jejuni* resistant birds produce B cell mediated response to clear the infection Connell *et al*. 2012 Possibly a full micro array would be beneficial to pin point exact interaction between *C. jejuni* and its avian host and compare the results observed in wild-type and mutant strains.

Future studies.

Bacteria-host interaction is an active process changes in the environment are picked up by bacteria and would lead to change in surface expression various molecules as host provides a “stressful environment” (Lebeer *et al.* 2010), the reversible changes in gene expression are common in bacteria, including *C. jejuni* (Tan et al. 2015). It has been identified that variation in rowth temperature (37°C and 42°C) can influence *Campylobacter* metabolism (Stintzi, 2002; Khanna et al., 2006; Young et al., 2007; Baserisalehi and Bahador, 2011) and LOS production (Semchenko et al., 2010).

Additionally cross-talk between carbohydrate modification pathways of *C. jejuni* may have additional role (Guerry and Szymanski 2008), identifying genes involved during colonisation may aid in identifying factors important in the clearance of *C. jejuni* by chickens, using a ligated-loop model would have allowed collection of both bacterial for analysis and collection of chicken cells involved in the response and observe the presence of cell types involved in detection of wild-type and mutant strains.

Conclusion

This project has, hopefully given a deeper insight into the processes involved in detection of *C. jejuni* by the immune system of chickens in addition to expanding our understanding of importance of *O*-glycosylation of flagella.

Immune recognition and manipulation of responses by *C. jejuni* will need a further investigation, from both, host- and bacterial point of view.

Chapter 6 - References

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Appendix

Composition of buffers and solutions

Buffer composition Endo free maxi kit

Buffer P1 (resuspension buffer)	50 mM Tris·Cl, pH 8.0; 10 mM EDTA; addition of 100 µg/ml RNase A
Buffer P2 (lysis buffer)	200 mM NaOH, 1% SDS (w/v)
Buffer P3 (neutralization buffer)	3.0 M potassium acetate, pH 5.5
Buffer QBT (equilibration buffer)	750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v); 0.15% Triton® X-100 (v/v)
Buffer QC (wash buffer)	1.0 M NaCl; 50 mM MOPS; 15% isopropanol (v/v), pH 7.0.
Buffer QN (elution buffer)	1.6 M NaCl; 15–25°C 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)
TE	10 mM Tris·Cl, pH 8.0; 1 mM EDTA

Gel extraction

Buffer EB - 10 mM Tris·Cl, pH 8.5

PBS buffer

Dissolve 8g NaCl, 0.2g KCl, 1.44g Na2HPO4 and 0.24g KH2PO4 in 800ml of distilled water. Adjust to pH 7.4 with HCl. Add water to 1 liter. Dispense into aliquots. Sterilize by autoclaving.

FACS buffer

PBS/BSA/Az

2X Rapid Ligation Buffer, T4 DNA Ligase (provided)

60mM Tris-HCl (pH 7.8)

20mM MgCl2

20mM DTT

2mM ATP

10% polyethylene glycol

TAE (50X)

242g Tris base

37.2g Na2EDTA•(2H2O) in 900ml of deionized water.

Supplemented with 57.1ml of glacial acetic acid, and adjusted the final volume with water to 1 litre.

BigDye® dilution buffer

250mM Tris-HCl (pH 9.0)

10mM MgCl

X-Gal

Dissolve 100mg X-Gal in N, N'-dimethylformamide to a final volume of 2ml. Dispense into 500µl aliquots, and store protected from light at –20°C. The final concentration of X-Gal is 50mg/ml.

IPTG

Dissolve 1.2g IPTG (isopropyl-β-d-thiogalactopyranoside) in deionized water to a final volume of 50ml. Filter sterilize (0.2µm), and store in 5ml aliquots at –20°C. The final concentration of IPTG is 0.1M

Agarose gel sample buffer (6X)

Dissolve 4g sucrose and 2.5mg bromophenol blue in 6ml of TE buffer [10mM Tris-HCl (pH 8.0), 1mM EDTA]. Once dissolved, bring up to a final volume of 10ml with TE buffer

0.5M EDTA (pH 8.0)

Add 186.1g disodium ethylenediamine tetraacetate•2H2O to 800ml of water. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH. EDTA will slowly go into solution as the pH approaches 8.0. Dispense into aliquots, and sterilize by autoclaving.

1M dithiothreitol (DTT)

Dissolve 3.09g DTT in 20ml of 0.01M sodium acetate (pH 5.2). Sterilize by filtration. Dispense into 1ml aliquots, and store at –20°C.

Versene (Gibco®)

This is EDTA solution for use as a gentle non-enzymatic cell dissociation reagent. Versene Solution (0.48 mM) is formulated as 0.2 g EDTA (Na4) per liter of Phosphate Buffered Saline (PBS).

Accutase

100 ml, ready to use, frozen sterile liquid. 1X ACCUTASE enzymes in Dulbecco's PBS (0.2 g/L KCl, 0.2 g/L KH2PO4, 8 g/L NaCl, and 1.15 g/L Na2HPO4) containing 0.5 mM EDTA·4Na and 3 mg/L Phenol Red.

10X PCR Buffer (Invitrogen *Taq* Cat: 18038-034)

200 mM Tris-HCl (pH 8.4)

500 mM KCl

Bacteria counting kit

Saline (NaCl) 0.15 M, 1 ml of 5 M NaCl in 29 ml of H_20

5% LB in Saline – 2.5 ml of LB broth (see above) into 47.5 ml of 0.15 M NaCl

MULTI-CORE™ 10X Buffer 250mM Tris-acetate (pH 7.8 at 25°C), 1M potassium acetate, 100mM magnesium acetate and 10mM DTT.

SOB Medium (per litre)

20.0 g of tryptone

5.0 g of yeast extract

0.5 g of NaCl

Deionised water was added to a final volume of 1 L then autoclaved

Filter-sterilised 1M $MgCl_2$ (10 ml) and 1 M $MgSO_4$ were added prior to use.

SOC medium (per 100 ml)

Filter-sterilised 2 M glucose (1 ml) was added to SOB (see above) to a final volume of 100 ml.

LB Agar (per Litre)

10 g of NaCl

10 g tryptone

5 g of yeast extract

20 g of agar

Deionised water was added to make a final volume of 1 L.

pH adjusted to 7.0 using 5 N of NaOH and then autoclaved.

Ampicillin 100

Dulbecco's Modified Eagle Medium (DMEM)

With 4500 mg/L glucose, L-glutamine and sodium bicarbonate and with pyridoxine

(substitutes pyridoxine HCl for pyridoxal HCl)

Supplemented with 10% heat-inactivated foetal calf serum (Gibco), 1% L-glutamine, 1% non-essential amino acids.

RPMI-1640

With 25 mM HEPES and sodium bicarbonate,

Supplemented with: 1% L-glutamine, 1 U/ml of penicillin and 1 µg/ml of streptomycin

10% chicken serum (for DC cultures)/10% foetal bovine serum (PAA, for macrophages),

Mueller-Hinton broth

Typical Formula	**gm/litre**
Beef, dehydrated infusion from	300.0

Casein hydrolysate	17.5
Starch	1.5

pH 7.3 ± 0.1 @ 25°C